CELL GROWTH PARAMETERS AND SISTER CHROMATID EXCHANGE LEVEL IN FIBROBLASTS FROM A PATIENT DIAGNOSED WITH BLOOM SYNDROME

A Thesis

Presented to

The School of Graduate Studies

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

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by

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September 1985

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An abstract of a Thesis by
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September 1985
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The problem. A patient, KB, diagnosed with the autosomal recessive genetic disorder Bloom Syndrome on the basis of clinical features, had failed to display the characteristic cytogenetic features of the syndrome. This research compared the growth response to bovine pituitary fibroblast growth factor, the mitotic index, and the sister chromatid exchange level of the KB fibroblasts with normal and known Bloom Syndrome control fibroblasts.

Procedure. The growth responses of the KB, normal, and Bloom Syndrome cell lines to fibroblast growth factor were determined by culturing the fibroblasts over a seven-day period in the presence of three concentrations of fibroblast growth factor in medium supplemented with two levels of fetal bovine serum. Cell concentrations were measured at regular intervals over the culture period using a Coulter counter. Sister chromatid exchange levels were determined by culturing the fibroblasts for 72 hours in medium supplemented with the thymidine analog 5-bromo-2'-deoxyuridine. Following culture, cells were harvested and stained for differentiation of the sister chromatids using a fluorescence plus Giemsa technique. A mitotic index for each cell line was determined by counting the number of cells in division per 1000 cells on the slides prepared for sister chromatid exchange analysis.

Findings. Like the normal cells, the KB fibroblasts utilized fibroblast growth factor for growth based on measurements of the population doubling time in the first 48 hours of culture. Unlike either of the control cell lines, however, the KB fibroblasts exhibited no response to fibroblast growth factor as measured by maximum cell concentration attained. The mitocic index for the KB cell line was considerably lower than that determined for either control line. The sister chromatid exchange level seen in the KB fibroblasts corresponded with that seen in the normal control fibroblasts and was unlike the elevated level seen in the Bloom Syndrome cells.

Conclusion. The diagnosis of Bloom Syndrome in the patient, KB, is not supported on the basis of the growth responses of the patient's fibroblasts to fibroblast growth factor, the mitotic index, and the sister chromatid exchange rate.

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INTRODUCTION

Bloom Syndrome: Features. Bloom Syndrome (BS) is a rare autosomal recessive genetic disorder that is characterized by both clinical and cytogenetic features. First identified as a syndrome by Dr. David Bloom in 1954 (Bloom 1954), the disorder has become a model for the study of growth, carcinogenesis, and the mechanisms of sister chromatid exchange (Passarge 1983).

Clinical features of BS include pre- and postnatal growth retardation that results in proportional dwarfism; a telangiectatic erythema consisting of a butterfly-shaped rash on the face; dolichocephaly, with an elongated characteristic facial appearance; immunodeficiency; and a predisposition to the development of cancers (Passarge 1983; Kaiser-McCaw 1982; Schroeder 1982). BS patients typically have normal intelligence. Other, more variable, symptoms include hypogonadism, and the appearance of "twin spots", adjacent areas of hyper- and hypopigmentation. A variety of additional congenital malformations occur with a slightly higher frequency in BS, but there is no pattern. Such abnormalities include congenital heart disease, annular pancreas, abnormal thumb, absent toe, and cryptorchidism. Many BS individuals have poor speech development, with high-pitched, squeaky voices. They may also exhibit characteristic behavior patterns such as restlessness,

irritability, and lack of concentration (Passarge 1983). Increased endocrinopathy and muscle weakness with age have also been reported (Ahmad et al. 1977). Growth retardation is perhaps the most important clinical feature of the disease (German et al. 1979). Birth size is small, with a mean birth weight of 1960 g (vs. a normal mean birth weight of 2500 g) and average length at birth is 44 cm (vs. a normal average length of 50 cm). Growth is below the third percentile throughout childhood, but the rate of growth is comparable to normal; however, a growth spurt before puberty is lacking or minimal in BS individuals (Passarge 1983). Average adult height of BS patients is 4'9" with normal body proportions. Sun sensitivity is a hallmark of the disorder, but skin areas other than the face, hands, and forearms are not unduly sensitive to sunlight and lesions produced in sensitive areas may show improvement with age. Skin lesions typically are less severe in females. This variability in skin sensitivity has made some cases of BS difficult to diagnose (Vanderschueren-Lodeweyckx et al. 1984). The characteristic immunodeficiency results from a decrease in the serum concentration in at least one class of immunoglobins (IgG, IgA, or IgM), with BS patients exhibiting weak levels of humoral and cellular responses after antigenic challenge (Weameas et al. 1979; Taniguchi et al. 1982). Bloom Syndrome infants frequently suffer from

feeding problems, and upper respiratory tract infections are common in BS children as a result of this immunodeficiency.

Cancer was first recognized by James German (1969) as being more prevalent in Bloom Syndrome patients. Approximately one cancer has occurred for every four persons diagnosed with BS since the syndrome was first described, with acute leukemia being the most common neoplasm (German 1980). Cancer in Bloom Syndrome is impressive in three ways: by the frequency of its occurrence, by the diversity of its type, and for the unusually early age of onset. Many of the cancers that occur with regularity in the general population just occur more commonly in BS and at an earlier age. Cancer deaths are over two times greater in BS individuals than in the general population; therefore, Bloom Syndrome is considered a cancer-prone syndrome.

In 1965 the first chromosome analysis was performed on the lymphocytes of a BS patient (German et al. 1965). This led to the discovery of the characteristic chromosome instability now associated with the disorder and opened the door for further cytogenetic analysis of Bloom Syndrome. Bloom Syndrome is now known as one of the "chromosome-breakage syndromes", a term coined by James German in 1965 (German et al. 1965) to draw attention to those disorders in which untreated lymphocyte cultures show an abnormally high frequency of structural chromosome aberrations. Cytogenetic

features seen in Bloom Syndrome include an increased number of chromosome breaks, gaps, and rearrangements, the presence of quadriradial configurations, and a greatly increased frequency of spontaneous sister chromatid exchange (SCE) (Chaganti et al. 1974, German et al. 1977a). Signs of chromosome breakage and reunion are evident in 5-15% of BS metaphases (Ray and German 1983) and quadriradial exchange formations between homologous chromosomes are seen in approximately 1% of BS metaphases (Passarge 1983). It is the elevated sister chromatid exchange level, however, that is the most important cytogenetic feature of the disease, as Bloom Syndrome is the only known genetic disorder in which the level of SCE is increased spontaneously. Normal cells show an average of 6.9 SCE's per metaphase, whereas in Bloom Syndrome cells, the SCE level is increased ten- to fifteenfold, for an average of 60-80 SCE's per metaphase (Chaganti et al. 1974; Bartram et al. 1975; German et al. 1977a; Dicken et al. 1978). These SCE's, as well as any chromosome breaks or gaps, occur in all regions of the genome and they are seen in all tissues (lymphocytes, fibroblasts, and bone marrow) (Ray and German 1983). The elevated SCE level has become such a hallmark of the disorder that it is now considered necessary to confirm the diagnosis of Bloom Syndrome (German et al. 1977a).

In 1965, the Mendelian basis of inheritance of Bloom Syndrome was established (German et al. 1965). The autosomal recessive nature of the disorder was suggested by the observations of German and co-workers that in some families more than one sibling was affected; that several incidences of consanguineous parentage were recorded; and about half the known cases were in Ashkenazi Jews with recent ancestry in the central European area. Following this mode of inheritance, the offspring from the union of heterozygotes for the mutant BS gene would have a 25% risk of having BS. Since its description more than 30 years ago, Bloom Syndrome has been diagnosed in over 100 persons. It has been observed in different parts of the world and in different ethnic groups, but remains rare except in the Ashkenazi Jewish population, which has been estimated to have a heterozygote frequency of 1:120 (German et al. 1977b, German et al. 1984). The sex ratio of males to females in BS is 1.4:1 (German et al. 1984). The reason for this imbalance is unknown; however, underdiagnosis in females is postulated. The current Bloom Syndrome population as a whole has a mean age of 18.2 years (range: 4-37). It is thought that this mean population age may be lowered by immunodeficiency as well as by the attrition rate through cancer deaths (German et al. 1984).

Hypermutability in Bloom Syndrome. The question of genetic heterogeneity remains unanswered in Bloom Syndrome, with no strong clinical or experimental evidence to support it. An unexpected and interesting finding in Bloom Syndrome research, however, has been the identification of two lymphocyte populations with respect to sister chromatid exchange in some BS individuals, in which the larger population displays the characteristic elevated SCE level, but a minor population (ranging from a few cells to 47%) shows SCE's in the normal range (German et al. 1977a; Shiraishi et al. 1983b). This dimorphism is not seen in dermal fibroblasts or in bone marrow, but has been seen in lymphoblastoid cell lines derived from single B lymphocytes (Shiraishi et al. 1983a). Such cell lines have either a normal or an elevated SCE rate and imply the existence of two cell populations in vivo. The reason for the two populations is unclear. It has been postulated that a back mutation has occurred at a BS locus which could restore normal gene function in one BS allele, giving rise to a heterozygous condition in that particular clonal line. The support for this comes from two in vitro studies (Warren et al. 1981; Vijayalaxmi et al. 1983) that indicate that spontaneous mutation frequency in BS fibroblasts may be ten times greater than in normal fibroblasts. Evidence also points toward a marked elevation in the in vivo spontaneous

mutation rate in BS PHA-stimulated lymphocytes (Ray and German 1983), lending further support. Warren et al. (1981) tested BS cells and found them to be spontaneously hypermutable. They showed BS fibroblasts to have an increase in the rate of spontaneous mutation at the HGPRT locus five to ten times that of control fibroblasts, and postulated that BS may be a mutator mutation, a mutation at a locus which maintains the rate of spontaneous mutation at an acceptably low level (Warren 1981). Such a phenomenon, seen in many organisms, is previously unrecognized in humans. Warren suggested that this possible mutator activity in BS can be correlated with the low birth weight and small stature in Bloom Syndrome individuals as the rate of growth fundamentally is determined by cell number. Cell loss, slow mitotic rate, or both, could account for these symptoms.

Theories on the origin of neoplasia in Bloom Syndrome. It is suggested that the increase in the incidence of malignancy in BS individuals could be explained by the somatic mutation theory of cancer (Warren 1981). Most human neoplasms consist of clonal proliferations of cells with chromosome abnormalities. Such chromosome abnormalities have been shown to be caused by environmental agents which cause cancer and are known to occur spontaneously in Bloom Syndrome cells. Currently held views

of mutation describe gene mutations and chromosome mutations (aberrations) as the ends of a common spectrum of genetic instability (Chaganti 1983). If chromosome breaks represent one point on such a spectrum, then the spontaneous mutation rate would be expected to be elevated in BS cells, a prediction which has been demonstrated. Warren (1981) proposes that in a hypermutable system such as Bloom Syndrome, enhanced mutation is the pathway to malignancy and, in general, can serve as a model for the origin of neoplasia. In Bloom Syndrome it is interesting to note that the majority of cancers are found in rapidly dividing tissues such as bone marrow and the GI tract (German 1983a). Inasmuch as the yield of spontaneous mutants is correlated with the number of cell divisions, this is as expected, as one would find the greatest number of cancers in those cells with the highest frequency of mutations. Transformation experiments, however, have failed to lend support to this theory. Bloom Syndrome cells are surprisingly less prone to viral transformation (Lin and Alfi 1980) and have shown no correlation between transformation and chromosome aberrations (Webb and Harding 1977; Lin and Alfi 1980; Shiraishi et al. 1983a), suggesting that chromosome aberrations are not directly related to the high cancer risk. Somatic cell recombination has also been proposed as a mechanism to explain the increased evidence of

neoplasia in Bloom Syndrome. This theory holds that a cell that contains a mutation at just one allele can, through somatic recombination, become homozygous for that mutation, and that in BS, twin spots may be a manifestation of just such an event (Festa et al. 1979; German 1983a). This supports the idea that cancer may be a recessive disorder at the cellular level.

Sensitivities of Bloom Syndrome cells. Bloom Syndrome cells, like normal cells, are sensitive to various mutagens in vitro, but the only hypersensitivities determined so far for BS cells are to ethylating agents such as ethyl methanesulfonate (EMS), as measured by SCE induction (Krepinsky et al. 1979) and to mitomycin C (MMC) as measured by cell death (Hook et al. 1984). Krepinsky et al. (1979, 1982) showed that the ethylating agent EMS induced six times more SCE's in BS lymphocytes than in normal or BS heterozygote lymphocytes at an EMS concentration of 3×10^{-4} M. In BS individuals with two lymphocyte populations, only the population with a high SCE frequency showed an increased sensitivity to EMS. EMS has no abnormal effect on BS cells with respect to cell death (Hook et al. 1984). This is the converse of the reactions of BS cells to the bifunctional alkylating agent mitomycin C, in which recent studies show a 55% increase in MMC-induced cell death of BS cells, but no hypersensitivity with

respect to sister chromatid exchange (Hook et al. 1984). Earlier research concerning MMC-induced SCE in Bloom Syndrome cells was contradictory. As MMC was shown to cause an increase in SCE in BS cells approximately two times the untreated frequency, it was thought that this reflected a hypersensitivity. The increase in MMC-induced SCE in normal cells, however, was found to be even greater (Shiraishi and Sandberg 1978, 1979, 1980), indicating that perhaps the effect of MMC was the same on both BS and non-BS cells, with the BS cells reaching a saturation level of SCE.

With respect to UV light, there has been a heterogeneity of response. The sensitivity of BS fibroblasts varies from strain to strain with exposure to far-UV light (wavelength below 290 nm), as measured by cell survival (Ishizaki et al. 1981; Hook et al. 1984), with the majority of strains tested showing normal sensitivity (Krepinsky et al. 1980). BS fibroblasts exposed to near-UV light (313 nm), however, have been found to have abnormal survival properties (Zbinden and Cerutti 1981) and excessive single-strand DNA breakage in six of eight BS strains tested (Hirschi et al. 1981). Near-UV light sensitivity is a more accurate measure of true BS cellular sensitivity under natural conditions as far-UV light is eliminated from the solar spectrum by the upper atmosphere.

Nature of the defect in Bloom Syndrome. Early theories held that Bloom Syndrome was very likely a repair-deficient syndrome, as chromosome aberrations, mutagen and solar sensitivities, and an increased cancer incidence are consistent with defects in DNA repair mechanisms. Research has shown, however, that BS cells respond normally in excision repair (Ahmed and Setlow 1978; Henderson and Ribecky 1980), post-replication repair (Shiraishi and Sandberg 1977), and normal repair of single-stranded breaks (Vincent et al. 1978; Schonberg and German 1980; Fornace et al. 1980) and double-stranded breaks (Hurt et al. 1981). The excision repair system was tested following damage by UV light and N-acetoxy-2-acetylaminofluorene (AAAF) and was found to be normal as measured by the rate of unscheduled DNA synthesis (UDS) following treatment (Ahmed and Setlow 1978; Remsen 1980). Research showed that BS fibroblasts had higher than normal levels of UDS (19-29% higher), but that the rate of UDS did not differ from that of normal controls following exposure to UV radiation (Giannelli et al. 1981). This finding could be explained by a greater than normal number of sites which need to be repaired initially on the BS DNA molecule. These researchers also hypothesized that BS cells may contain a diffusable factor which influences at least the initial rate of UDS, as evidenced by fusion experiments. The general trend of these studies has

seemed to indicate that DNA repair is intact in Bloom Syndrome. Further efforts to elucidate the basic defect have measured the levels of DNA polymerases (α, β, and γ) and apurinic site-specific endonuclease in BS cells and found them to be normal (Parker and Lieberman 1977; Moses and Beaudet 1978; Bertazonni et al. 1978). It was also found that all four deoxyribo- nucleoside triphosphate pool levels correspond to those of normal controls (Warren 1981).

Most recent research has focused on the hypothesis that Bloom Syndrome is a DNA-replication-deficient syndrome (Giannelli et al. 1977). DNA replication in mammalian cells is initiated at many different sites on the same molecule in units called replicons. Synthesis is bidirectional, proceeding outward from each initiation point, an action termed replication fork movement. Termination occurs when two adjacent replicons join and the daughter DNA molecules separate. The DNA replication rate is determined largely by two components: the number of actively replicating replicons, and the average linear fork movement rate. BS cells have shown normal replicon unit length and incidence of bidirectional replication (Hand and German 1977; Giannelli et al. 1977). Cells from patients with BS, however, are apparently the only human cells that have a slower than normal replication fork movement rate (German et al. 1979; Kapp and Painter 1982). It has been found that

fork displacement rates in BS cells are approximately 30% slower than those in normal cells, with a more severe effect seen in lymphocytes than in fibroblasts (Kapp 1982; Hand and German 1977). German et al. (1979) reported that the rate of DNA chain maturation in BS cells is lower than that in normal cells and many researchers have reported that BS fibroblasts grow more slowly than normal fibroblasts (Lechner et al. 1983; German et al. 1984). Brat (1979) found the BS cell cycle duration to be similar to that of a normal cell line, but reported a lowered mitotic index. In culture, BS cells have been shown to exhibit less than 1% clonal efficiency, in contrast to a greater than 10% clonal efficiency for non-BS cultures (German et al. 1984). Measuring lifespans of fibroblasts in terms of number of passages, Thompson and Holliday (1983) found that BS fibroblasts had a lifespan of 27.8 passages, approximately half that of controls. This could be due to an impaired ability to respond fully to growth stimulation, as evidenced by the results of a study of Lechner et al. (1983) in which the response of BS cells to epidermal growth factor (EGF)-promoting activity was half that of normal cells. Ockey (1979), in a contradictory study, looked at the relationship between DNA chain growth and the distance between adjacent replicons in BS fibroblasts and found the mean rate of chain growth was decreased only when cells were grown at a low

density and concluded that the slow rate of chain growth in BS cells is an artifact introduced by culture conditions.

Co-cultivation and cell hybridization experiments. In an attempt to answer the crucial question of whether the increased frequency of sister chromatid exchange in BS cells is due to the lack of a normal metabolite or the production of an abnormal one, the co-cultivation of BS and non-BS cells has been used experimentally. The question is still controversial in view of contradictory reports. Tice et al. (1978) maintain that the rate of SCE in normal fibroblasts was increased (by 20-50%) above the normal level when co-cultivated with BS cells, as was the SCE frequency of PHA-stimulated normal human lymphocytes grown in medium "conditioned" for 48 hours by proliferating BS fibroblasts. They concluded that the chromosome instability in Bloom Syndrome results from the over-production of some DNA-damaging cellular component. Most attempts to corroborate these experiments by Tice et al. have been unsuccessful, although Barnabei and Kelly (1982) wrote of work corroborating Tice's findings. Emerit and Cerruti (1981) reported that greatly concentrated ultrafiltrates of BS-conditioned medium had a clastogenic and slightly SCE-inducing effect on normal PHA-stimulated lymphocytes. Other co-cultivation and conditioned medium experiments have yielded data that contrast with that of Tice et al. Van

Buul et al. (1978) demonstrated the suppression of SCE's by 30% in BS fibroblasts upon co-cultivation with Chinese hamster ovary (CHO) cells, but only if cell-to-cell contact occurred. They reported that the BS cells, in turn, had no effect on the CHO cells. Bartram et al. (1979), in the co-cultivation of normal and BS fibroblasts, showed a reduction of SCE's in the BS cells that was repeated with the use of normal-conditioned medium. These results suggest that normal cells produce an agent that acts as a corrective factor in BS cells, a factor either absent or inactive in Bloom Syndrome. These experiments have been duplicated and the results corroborated by other researchers (Rudiger et al. 1980; Schonberg and German 1980; Bartram et al. 1981). It has also been shown that BS heterozygote cells exhibit a corrective effect upon co-cultivation with BS homozygote cells, although to a lesser capacity, suggesting a gene dosage effect (Bartram et al. 1981). Rudiger (1982), in the co-cultivation of BS fibroblasts with normal, xeroderma pigmentosum, and Fanconi's anemia fibroblasts, produced a dose-dependent reduction in Bloom-specific SCE, the rate of SCE decreasing linearly with increased portions of non-BS cells (while the SCE rates in non-BS cells remained unchanged). He related this reduction to a soluble corrective factor (M.W. 10,000-20,000) which is produced in culture by non-BS proliferating fibroblasts. He postulated

that this corrective factor may represent a cancer-protective principle that is defective in BS individuals and noted that the finding of such a factor could be compatible with the previous findings of a clastogenic factor in BS (Tice et al. 1978) should the defect be due to a genetically defective detoxification system. To complicate the matter, however, Shiraishi et al. (1981) found the SCE frequencies of normal, BS heterozygote, and BS homozygote lymphocytes to be unchanged following co-cultivations at 1:1 ratios for 24 hours, and Ray and German (1983) reported that neither fibroblasts nor lymphocytes from normal individuals affected the SCE rate of BS fibroblasts or lymphocytes, even when co-cultivated at a non-BS:BS ratio of 4:1. They also showed that normal-conditioned medium was totally ineffective in reducing the SCE frequency in BS fibroblasts. The seemingly conflicting results of these co-cultivation experiments are hard to reconcile. In a series of cell hybridization experiments, an extension was seen of the findings of those co-cultivation experiments which suggested normal-cell production of a BS corrective factor. Complete correction of the mutant phenotype was reported, with a reversion of SCE's in BS fibroblasts to normal levels when hybridized with normal fibroblasts (Bryant et al. 1979; Shiraishi et al. 1981). Alhadeff et al. (1980) showed full correction of the elevated SCE level in BS fibroblasts upon hybridization

with CHO cells, concluding that increased SCE in Bloom Syndrome is due to the loss of a normal function that is restored by hybridization.

Detoxification deficiency theory. A current hypothesis that would explain the findings of Tice et al. (1978) and Emerit and Cerutti (1981) concerning the formation of a clastogenic factor (CF) in BS cells is based on the suspicion that BS cells are deficient in their ability to detoxify active oxygen species. Cerutti (1982) noted that some clinical, cellular, and biochemical abnormalities in Bloom Syndrome point toward disturbances in the formation or the detoxification of active oxygen species (superoxide radicals, singlet oxygen, hydroxyl radicals, hydrogen peroxide). His research showed that Cu-Zn-Superoxide dismutase (Cu-Zn-SOD), an enzyme which catalyzes the dismutation of superoxide radicals, strongly inhibits the activity of a clastogenic factor from BS fibroblasts. In light of this, plus the sensitivity of BS patients and their cultured fibroblasts to solar radiation, the increased frequency of sister chromatid exchange, and the increased cancer incidence, a model was put forth proposing the formation of a clastogenic factor by elevated levels of active oxygen species in BS cells (Emerit and Cerutti 1981; Cerutti 1982). Emerit et al. (1982) showed that the generation of superoxide radicals in growth medium increased

chromosome breakage and SCE rates in human lymphocytes, actions which were prevented almost entirely by SOD. Their data suggest a role for superoxide radicals in the origin of chromosome mutation. If true, an increase in the intracellular concentration of superoxide radicals could contribute to chromosome aberrations and a high rate of sister chromatid exchange in Bloom Syndrome. The induction of SOD is mediated by the substrate, so that an increase in SOD activity should reflect an increase in superoxide radical concentration. Nicotera et al. (1983) quantitated the activities of Mn- and Cu-Zn-SOD in two BS fibroblast lines (Mn-SOD is mitochondrial SOD; Cu-Zn-SOD is cytoplasmic SOD) and found a 45% increase in Cu-Zn-SOD activity and a 125% increase in Mn-SOD activity. They postulated that the superoxide radicals responsible for the induction of this SOD activity in BS may be at such high levels that the increased SOD was insufficient to detoxify them. There are several mechanisms for the formation to active oxygen species in mammalian cells, and an abnormality in their formation or detoxification would be expected to affect all tissues (Cerutti 1982).

Sister chromatid exchange. Sister chromatid exchange is a reciprocal interchange between DNA replication products that requires a means of differentiation of the sister chromatids for detection. The discovery of SCE was made by

Taylor et al. (1957) following the labeling of replicating chromosomes with ^{3}H-thymidine and the observation of the label's segregation. Taylor showed that when the label was incorporated into DNA during the first round of replication and analyzed at the second metaphase, the label was observed in only one of the sister chromatids of the chromosome. This delineation of the two chromatids allowed one to observe "occasional chromatid exchanges" (Taylor et al. 1957). Taylor concluded from his observations that SCE is a double-strand exchange, and that the rejoining of the strands is not random, but is restricted to subunits of the same polarity.

Use of the ^{3}H-thymidine label to detect SCE has given way to the use of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) as a label, which allows for higher resolution compared with autoradiography (Zakharov and Egolina 1972). In a variety of techniques, cells are grown in the presence of BrdU for two rounds of replication so that one chromatid is bifilarly substituted with the analog and the sister chromatid is unifilarly substituted. Differences in BrdU substitution can then be used to differentially stain the chromatids. Latt (1973) reported the method of staining chromosomes with the fluorescing bisbenzimidazole dye Hoechst 33258 so that the bifilarly substituted chromatid quenched the fluorescence to a greater

extent than did the unifilarly substituted chromatid. The procedure most widely used today in the detection of sister chromatid exchange is the "fluorescence plus Giemsa (FPG) technique" (Perry and Wolff 1974) in which Hoechst 33258 and short-wavelength light are used to treat the chromosomes, followed by staining with Giemsa. With this technique, the less-substituted chromatid is stained more intensively.

The observation that a significant portion of SCE's are independent of technical factors involved in their detection has led to the characterization of SCE's in normal cells (Zakharov 1982). A lower level of SCE's in heterochromatic regions of the chromosome as compared with the euchromatic regions has been observed (Hsu and Pathak 1976), and also the nonrandom distribution of SCE's among human chromosome groups B, E, F, and G (Latt 1974a). The average frequency of sister chromatid exchanges in normal cells was found to be 10±5 (Morgan and Crossen 1977). Chaganti et al. (1974) were the first to show a manyfold increase in the SCE levels in cells of Bloom Syndrome individuals above this baseline rate. Pioneering work by Latt (1974b) showed SCE as a sensitive indicator of mitomycin C damage in cells. Further research has shown that SCE's are produced by the majority of mutagenic carcinogens, although they have yet to be quantitatively correlated with mutations (Stetka 1982). Nevertheless,

SCE's are apparently manifestations of the interactions of chemical or physical agents with a cell's DNA, and research has expanded to the extent that now sister chromatid exchange is used extensively as a sensitive test for agents with mutagenic or carcinogenic potential.

SCE is difficult to understand on the molecular level, and several theories have been advanced to explain its mechanism. A number of these have proposed that sister chromatid exchange is a means of replication bypass that enables the DNA replication process to bypass any lesions or their repair intermediates that might otherwise inhibit or interrupt replication (Shafer 1977, 1982). Parental DNA strand incisions may occur via direct DNA crosslinks, single-strand breaks, and endonuclease incision. According to these models, SCE induction is linked to the bypass of these DNA lesions at the replication forks and, thus, is related to a cell's repair capacity. Ishii and Bender (1980) have put forth a "replication-detour model" which suggests that SCE occurs at the replication fork, but is due to reduced chain elongation or fork displacement rate. The most acceptable model to date is the Painter model (Painter 1980) which is based on the premise that double-strand breaks (one in each parental strand) occur frequently at the junctions of adjacent replicons during replication. This model is supported by recent evidence of DNA topoisomerases

that can unwind DNA by making and rejoining such double-strand breaks (Stetka 1982; Cleaver 1983). According to the model, a reduction in fork displacement rates should result in a situation in which four free termini from a completely replicated replicon cluster meet and oppose two termini of a non-replicated cluster. SCE would occur when the daughter strands of the duplicated cluster combine with the opposing cluster's parental strands of the same polarity. It accurately predicts that agents which reduce fork displacement rates such as MMC and UV light should efficiently induce SCE's and that a disorder such as Bloom Syndrome, which has a fork displacement rate 30% below normal, should have a high SCE frequency (Stetka 1982).

Growth of cells in culture. Mammalian cells in culture exhibit much the same classical growth kinetics as microorganisms (Paul 1975). When cells are taken from a stationary culture, there is at first a "lag" phase before growth commences. During this phase, there is generally little increase in cell number. It is, rather, a period of adaptation during which the cell replaces elements of the glycocalyx lost during trypsinization, attaches to the surface of the culture flask, and spreads out. It is during this lag phase that DNA and protein synthesis are initiated. Following this, growth of the culture proceeds steadily in what is known as the "logarithmic", or "log",

phase, when cell number increases exponentially. During this stage, the population doubling time, the time it takes for a culture to increase two-fold, can be determined (Freshney 1983). The length of the log phase can vary, depending on the seeding density, the growth rate of the particular cell strain, the density at which cell proliferation is inhibited for a particular strain, the availability of nutrients, pH, and temperature. During this time of proliferation, normal cells have an absolute demand for growth factors, provided in animal serum or otherwise, and will divide as long as free space is available and the growth factors are provided (Rudland et al. 1974; Westermark and Wasteson 1975). Toward the end of the log phase, the culture becomes confluent, when all available growth surface is occupied and all cells are in contact with surrounding cells. At this point, the growth rate of the culture is reduced and may cease. Entering what is known as the "stationary" or "plateau" phase, cells become less motile and may align themselves in parallel rows.

Cell growth kinetics have been used to compare differences in cell lines and differences in the responses of cell lines to various nutrients and other medium components. Such measurements include cell cycle time, plateau level, doubling time, and the mitotic index. Measurement of cell cycle time often employs radioactive

labeling to measure cell transit through one generation until its return to the same point in the cycle. The plateau level, the cell concentration at the plateau phase, is dependent on the cell type and the frequency of medium replenishment, but is difficult to measure accurately as a steady state. The measurement of the population doubling time is used to quantify the response of cells to different inhibitory or stimulatory culture conditions (Freshney 1983). Mitotic index, the percentage of cells in mitosis as a proportion of the whole population, is used as a measure of cell proliferation.

Fibroblast growth factor. Discovered by Gospodarowicz (1974), fibroblast growth factor (FGF) is a polypeptide isolated from the bovine pituitary and brain which enhances the proliferation of fibroblastic cells in monolayer culture (Katoh and Takayama 1984). FGF has been shown to provoke a resumption of growth in density-inhibited fibroblast cultures (Gospodarowicz et al. 1976) and to act as a mitogen for early passage human fibroblast cultures in the presence of low serum or no serum at all. In high concentration of serum (10% or greater) FGF was shown to have an additive effect over serum (Gospodarowicz et al. 1976). Gospodarowicz and Moran (1975) showed that FGF in culture medium with serum reduced the division time of human fibroblast cultures from two days to one day, with a final

cell density 3.3 times greater than that observed using optimal serum concentration alone. Pituitary FGF has been shown to be active at concentrations as low as 0.25 ng/ml when used to culture human endothelial cells (Gospodarowicz et al. 1983). Katoh and Takayama (1984) found FGF, in conjunction with 5% fetal calf serum, to stimulate human dermal fibroblasts dose-dependently at up to 200 ng/ml. Gospodarowicz and Moran (1975) tested the effect of FGF on slow-growing cells. When skin fibroblasts from patients with cystic fibrosis, whose cultured fibroblasts grow much more slowly than those from normal individuals, were grown in the presence of FGF with 10% fetal calf serum, FGF was shown to decrease their doubling time by 30-50%.

Bloom Syndrome: Two diagnosed cases. In central Iowa two brothers have been diagnosed as having Bloom Syndrome. Born to unrelated, non-Jewish parents, the brothers, ages 6 and 7, have been diagnosed by physicians at three separate medical facilities based on their clinical symptoms. KB, the older of the two boys and the object of this study, was born July 14, 1977, at full term with low birth weight dwarfism (2430 g). He has continued this dwarfism with normal body proportions. He is photosensitive and displays the telangiectatic erythema, or typical "butterfly" facial rash associated with BS. Since birth, he has shown a predisposition to infections, an immunological

feature of the disorder. In addition to these features, he exhibits dolichocephaly (elongated facial appearance), micrognathia (small jaw), clinodactyly (deflection of the fingers), and shows some livedo reticularis (a reddish-blue netlike mottling of the extremities). He has small, light-colored eyelashes, and a severe enamel defect of the teeth. There is no sign of mental retardation.

While the clinical features strongly support the diagnosis of Bloom Syndrome, the cytogenetic features fail to comply. The chromosomes from PHA-stimulated lymphocytes have been examined for both boys and show neither an increased frequency of sister chromatid exchange nor increased breakage. This lack of characteristic cytogenetic evidence to support the diagnosis could indicate genetic heterogeneity in Bloom Syndrome. Heterogeneity occurs when homozygosity at a single locus is able to produce an array of abnormalities that are recognized clinically as a single syndrome (German 1983b) or when mutations at different loci act independently to produce traits that are difficult to distinguish clinically (Thompson and Thompson 1980). Genetic heterogeneity is undocumented in Bloom Syndrome, although there has been a report of a previous diagnosis of BS in a Japanese child who had the clinical features of the disorder but a normal frequency of spontaneous chromosome aberrations (Arase et al. 1980). The report has also been

made of a patient with the cytogenetic features of Bloom Syndrome but without a history of sun-sensitivity (Uchiya et al. 1979). If the fibroblasts of the boys in question are shown to have an elevated frequency of spontaneous chromosome aberrations, this would lend support to the diagnosis and would be of interest in the establishment of a Bloom Syndrome variant.

The purpose of this study was to measure the sister chromatid exchange level in the fibroblasts of the diagnosed Bloom Syndrome patient, KB, and to compare it with the SCE levels in the skin fibroblasts of a confirmed Bloom Syndrome individual and a normal individual. A comparison was also made of the mitotic indices of the three cell lines. The study further investigated the growth response of the dermal fibroblasts of the KB patient, a known Bloom Syndrome control, and a normal control to various concentrations of fibroblast growth factor, administered in conjunction with either high or low concentrations of fetal calf serum. Growth response was measured in terms of population doubling time and maximum cell concentration attained.

METHODS AND MATERIALS

Cell lines. Dermal tissues from KB and the normal control, CS, were obtained by skin punch biopsies. These tissues were grown as primary explants to develop

fibroblastic monolayer cultures. Control Bloom Syndrome dermal fibroblasts (GM2548A) were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, New Jersey).

Culture conditions. All cell lines were routinely maintained in Eagle's minimal essential medium (MEM) (D-medium, Gibco) or Dulbecco's modified MEM (DME, Gibco) supplemented with 20% fetal bovine serum (Hyclone). Either gentamycin sulfate (50 ug/ml) (Sigma) or penicillin/streptomycin (100 units/100 ug/ml) (Gibco) was added as an antibiotic. Cells were maintained in plastic tissue culture flasks (Corning, 25 cm^2 or 75 cm^2) at 37°C in a 5% CO_2 atmosphere. Media was replaced twice a week. Cells were routinely passaged when they reached confluence. For passage or harvest, cells were washed with versene (0.02% EDTA in Puck's saline A), then removed from the surfaces of the culture flasks with 0.04% trypsin (Difco) in versene.

Growth response to fibroblast growth factor. To determine the growth response of each cell line to fibroblast growth factor, fetal bovine serum, and these two growth supplements in combination, cells were cultured in medium supplemented with either high fetal bovine serum (20%) or minimal fetal bovine serum (0.5%), and three concentrations of bovine pituitary FGF (0.1 ng/ml; 1.0 ng/ml; and 10.0 ng/ml) (Sigma). Final working

concentrations of FGF were obtained from dilutions of an FGF stock solution (100 ng/ml).

Cells from each of the cell lines (KB, CS, GM2548A) were seeded in eight sets of 25 cm^2 culture flasks (4 flasks/set) at a cell density of 1 x 10^6 cells/ml in 5 ml culture medium. FBS and/or FGF was added at the initial seeding (Day 0) as follows:

Set of Culture Flasks	Growth Supplement
A	Control
B	20% FBS
C	20% FBS, 0.1 ng/ml FGF
D	20% FBS, 1.0 ng/ml FGF
E	20% FBS, 10.0 ng/ml FGF
F	0.5% FBS, 0.1 ng/ml FGF
G	0.5% FBS, 1.0 ng/ml FGF
H	0.5% FBS, 10.0 ng/ml FGF

Fresh FGF was added daily to those flasks still in culture. Medium (with the appropriate serum concentrations) was replaced every other day (Days 2, 4, and 6).

Growth response was measured as the number of cells/ml over a seven-day period using a Coulter counter. For each cell line, the cell concentration in a flask with each of the eight treatments (A-H) was measured every other day (Days 1, 3, 5, and 7). Each of two samples from a flask was counted twice and these figures were averaged to determine number of cells/ml. Cell concentrations were plotted versus time in days to establish growth curves for the different growth supplement conditions.

Sister chromatid exchange analysis. Cells from each cell line were seeded in 25 cm^2 culture flasks at approximately 1 x 10^6 cells/ml in 10 ml DME medium supplemented with 20% fetal bovine serum and penicillin/streptomycin (100 units/100 ug/ml). 0.1 ml 10^{-2} M 5-bromo-2'-deoxyuridine (BrdU) (Sigma) was added to each flask for a final BrdU concentration of 30 ug/ml. Flasks were incubated at 37°C in a 5% CO_2 atmosphere. At 24 hours, both medium and BrdU were replaced. At 68 hours, colcemid (1 ug/ml) (Gibco) was added to block dividing cells in metaphase, and at 72 hours, cells were harvested. To harvest, cells were washed once with versene, then removed from the culture flask with 0.04% trypsin (Difco) in versene. Cells were suspended in fresh medium supplemented with 10% FBS to halt the action of the trypsin, and all culture fluids were combined in a 15 ml conical centrifuge tube. Cells were concentrated by centrifugation at 1000 rpm for 6 minutes, then treated with 10 ml 0.075 M KCl hypotonic solution for 10 minutes. Cells were fixed in Carnoy's fixative (3 methanol:1 glacial acetic acid) for 20 minutes, then stored overnight in fixative at 4°C.

Slides were made by dropping a concentrated cell suspension on a cold, wet slide which was then passed through a flame to dry. Slides were examined with a Zeiss photomicroscope II using phase contrast, and those slides

with metaphase spreads were aged at 60°C overnight.

To stain for chromatid differentiation and sister chromatid exchange analysis, slides were placed in a coplin jar containing a solution of the bisbenzimidazole dye Hoechst 33258 (5 ug/ml) in Sorenson's buffer, pH 6.8, for 15 minutes, washed in distilled water, then mounted in Sorenson's buffer, pH 8.0. Mounted slides were exposed to a high-intensity UV light at a distance of approximately 10 cm for 1-1/2 hours. Following this exposure, coverslips were removed and slides were washed in distilled water, then stained in 5% Giemsa solution (Gurr's R66) in Sorenson's buffer, pH 6.8, for 30 minutes.

Slides were examined with a Zeiss photomicroscope II. Metaphases exhibiting good differential staining and little or no overlap of chromosomes were considered acceptable for scoring of sister chromatid exchanges. Under oil immersion at 1250X, interchange points, as evidenced by differences in staining intensity along the chromatids, were counted. Each exchange point was counted as an SCE, rather than the area between exchange points.

Mitotic index. The mitotic index for each cell line was determined by counting the number of cells in division per 1000 cells from the slides prepared for sister chromatid exchange analysis.

RESULTS

Growth response of fibroblasts to growth supplement FGF. The growth responses of the fibroblastic cell lines KB, CS (normal fibroblasts), and GM2548A (Bloom Syndrome fibroblasts) to various concentrations of fibroblast growth factor with both high and low levels of fetal bovine serum were measured two ways. First, population doubling time, the time required for the number of cells in culture to increase by a factor of two, was used to compare growth rates among the cell lines. Second, maximum cell concentrations attained over the seven-day culture period by each cell line were compared.

Figures 1-8 show the growth curves of the fibroblastic cell lines (KB, CS, and GM2548A) under each of the eight different culture conditions (A-H). The curves are semi-logarithmic curves, i.e., the numbers of cells/ml are plotted on a logarithmic scale versus time on an arithmetic scale. This type of growth curve has the advantage of minimizing counting errors and furthers the possibility that population doubling time can be expressed as a function of the slope of the straight-line portion of the curve. Cell counts were made by taking 0.5 ml of a 1:500 dilution from a 5.0 ml cell suspension. This required that Coulter counter readings be multiplied by 5,000 to achieve a final determination of number of cells/ml in the

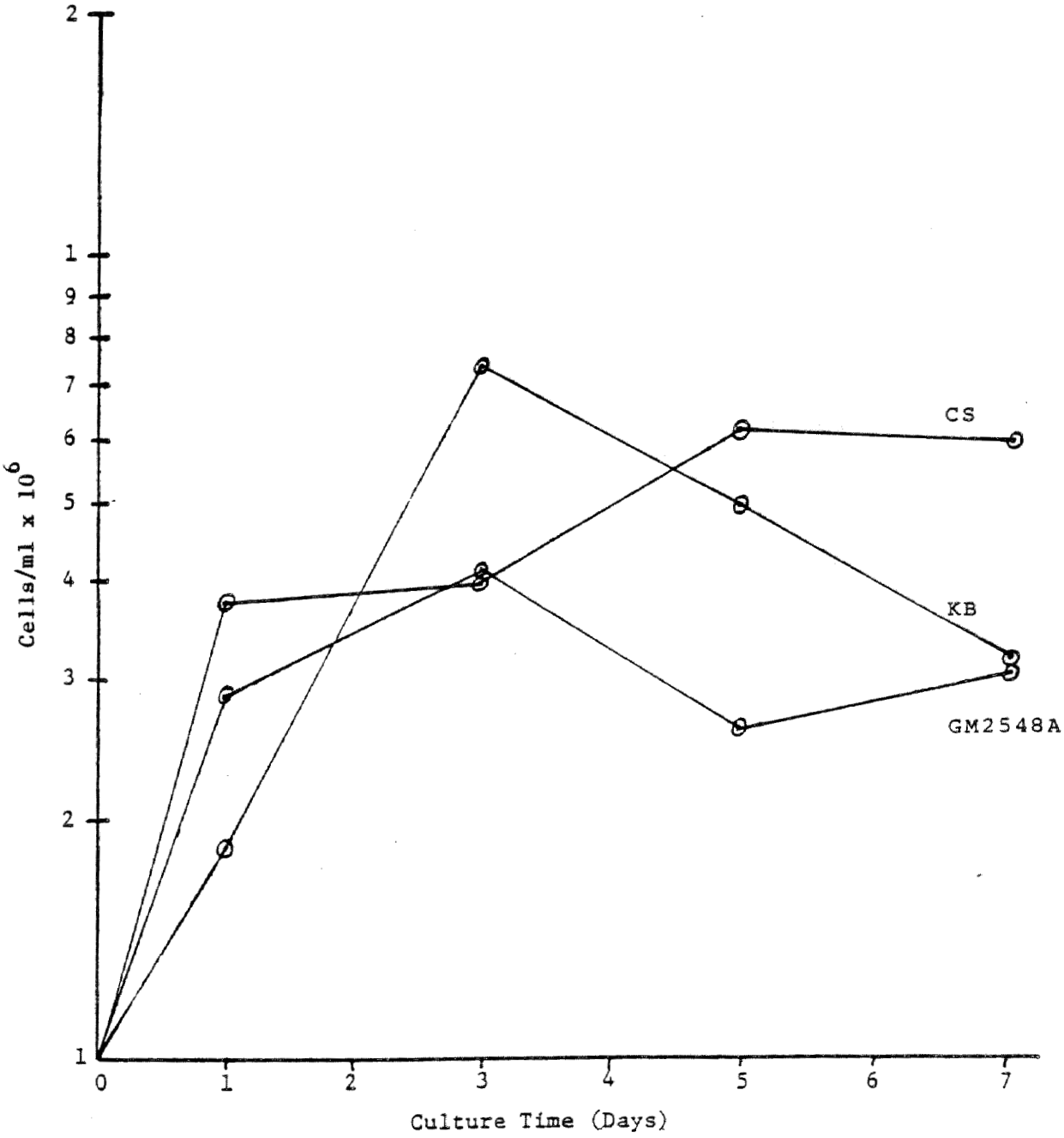

Figure 1. Fibroblast growth curve, Treatment A, Control (no growth supplement).

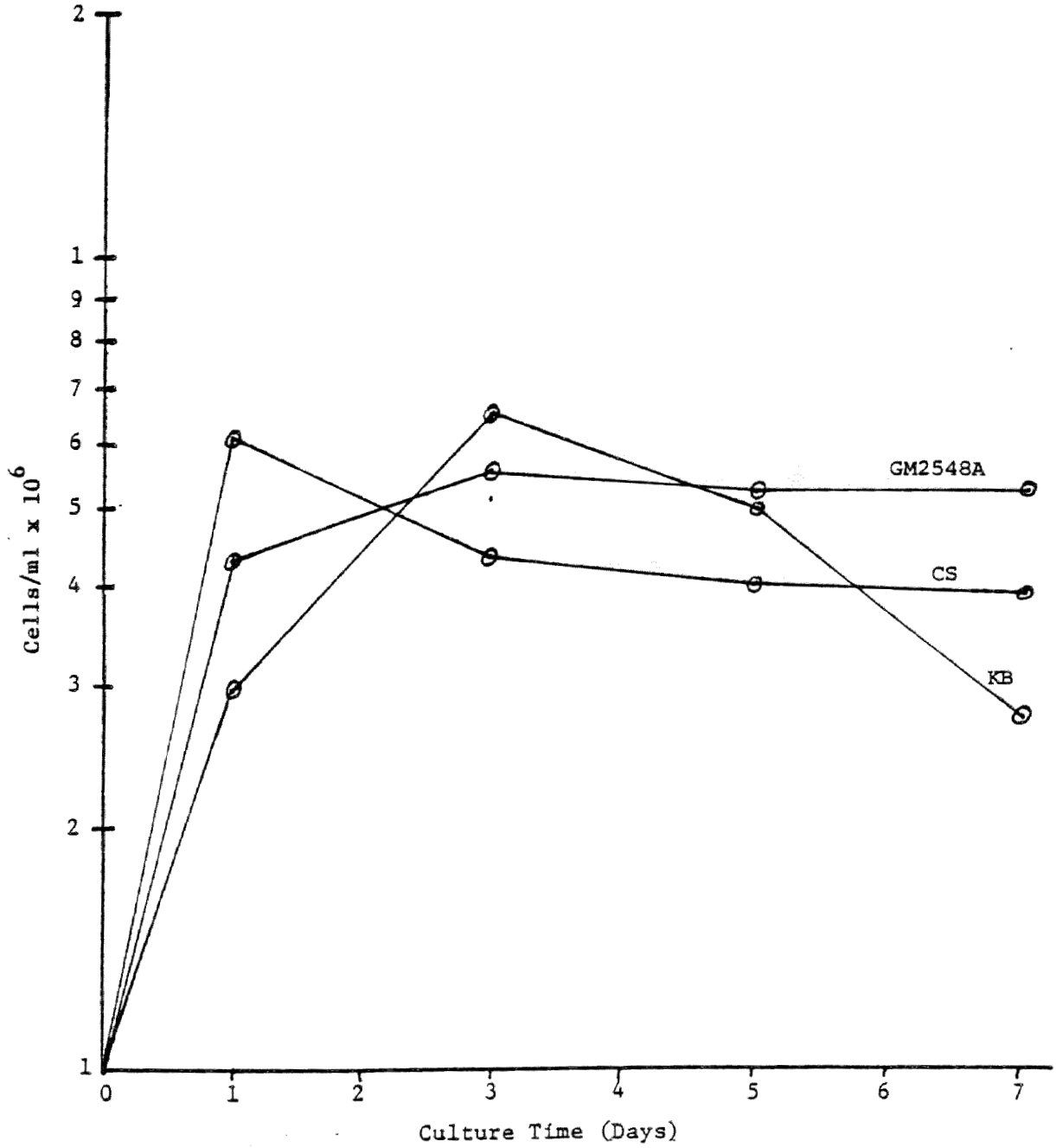

Figure 2. Fibroblast growth curve, Treatment B, Growth supplement: 20% fetal bovine serum.

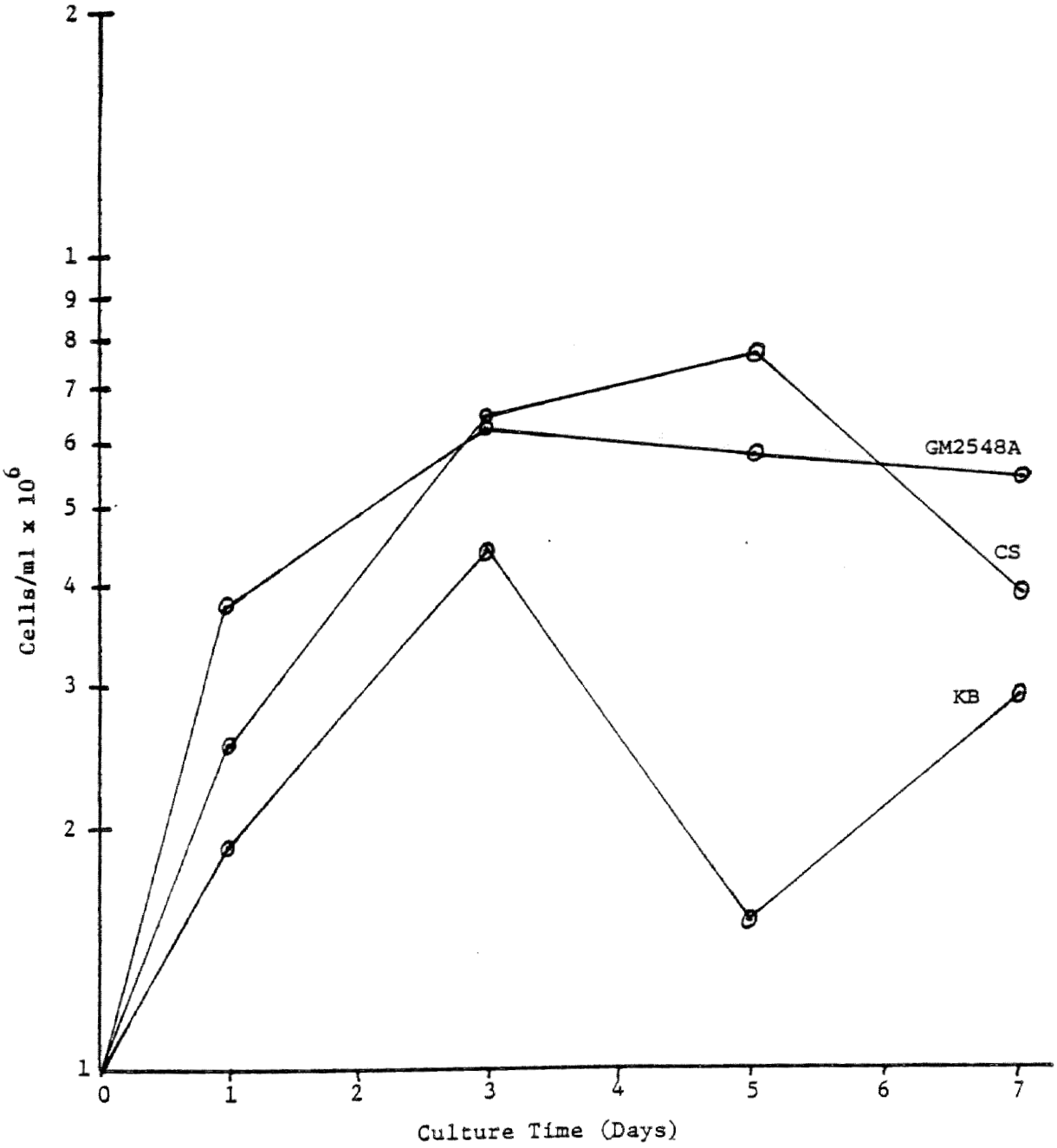

Figure 3. Fibroblast growth curve, Treatment C, Growth supplements: 20% fetal bovine serum, 0.1 ng/ml fibroblast growth factor.

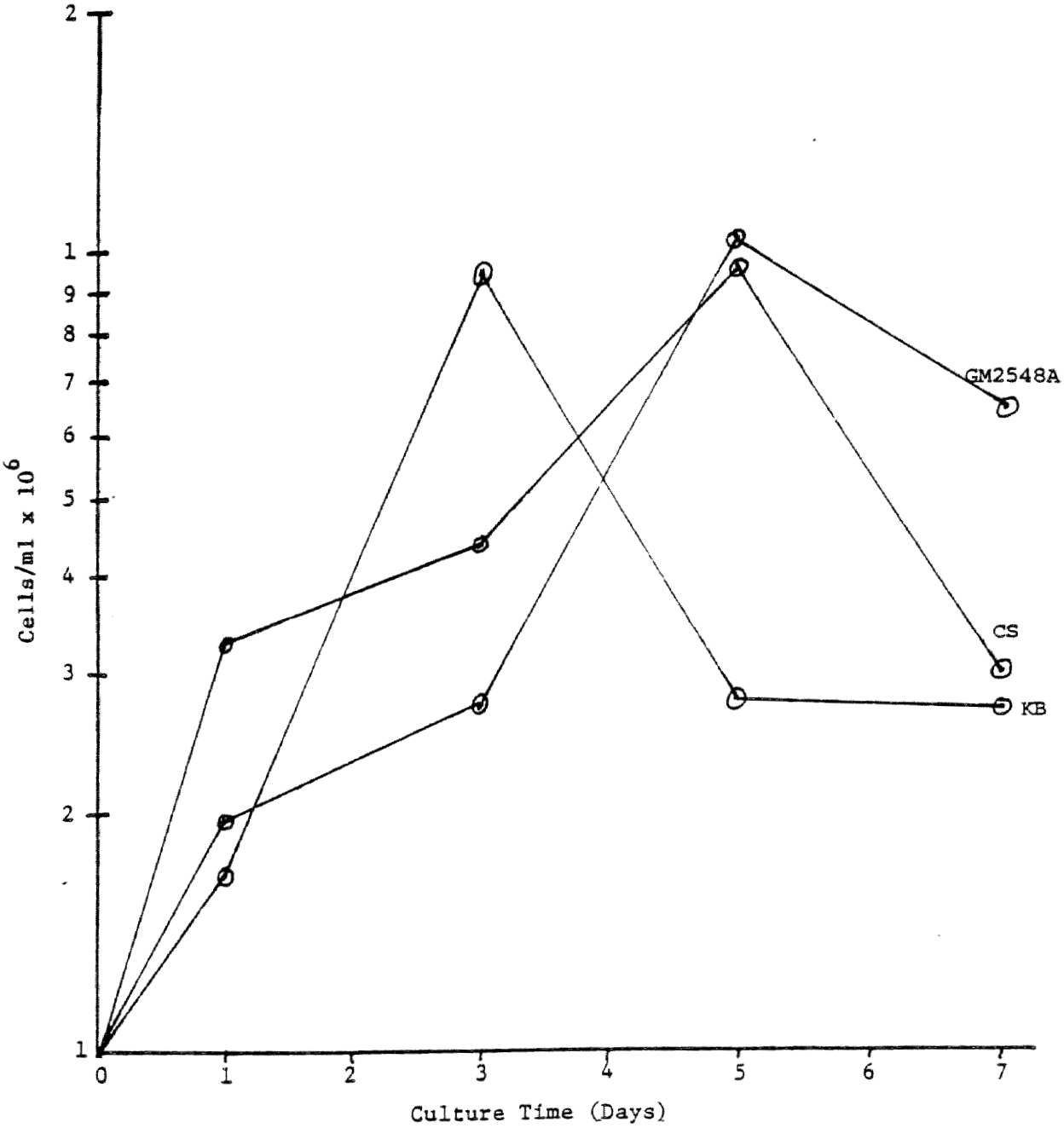

Figure 4. Fibroblast growth curve, Treatment D, Growth supplements: 20% fetal bovine serum, 1.0 ng/ml fibroblast growth factor.

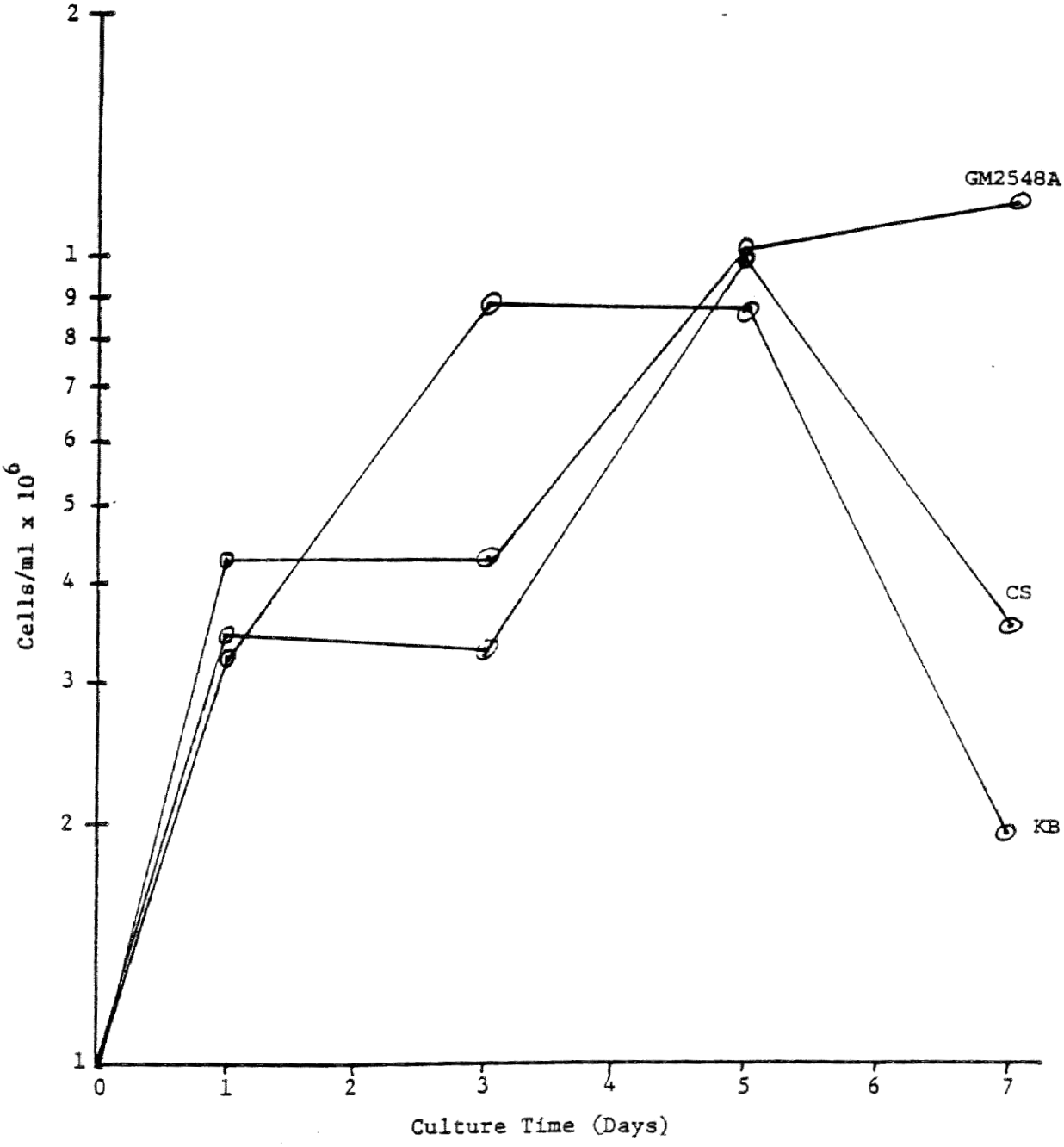

Figure 5. Fibroblast growth curve, Treatment E, Growth supplements: 20% fetal bovine serum, 10.0 ng/ml fibroblast growth factor.

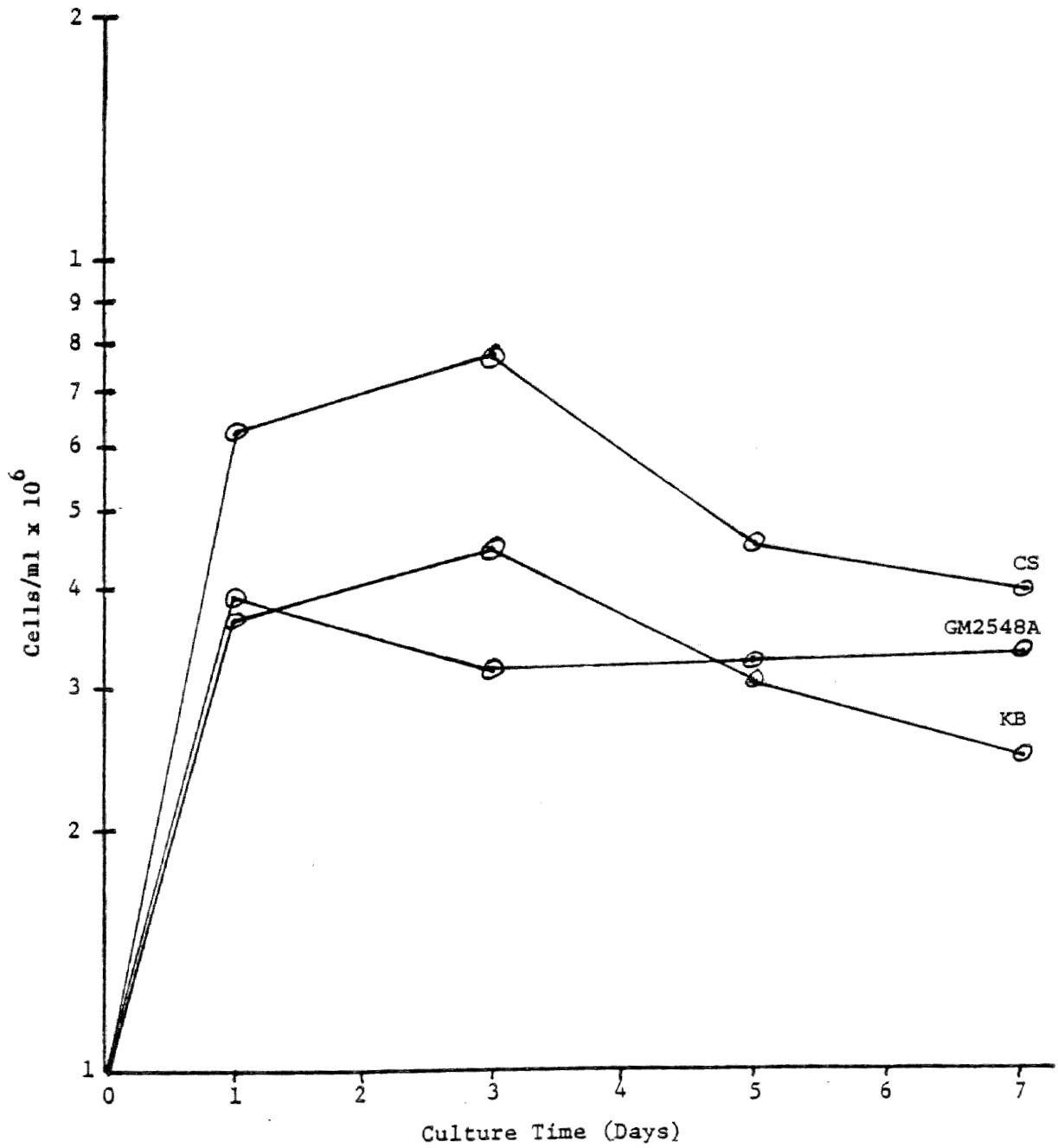

Figure 6. Fibroblast growth curve, Treatment F, Growth Supplements: 0.5% fetal bovine serum, 0.1 ng/ml fibroblast growth factor.

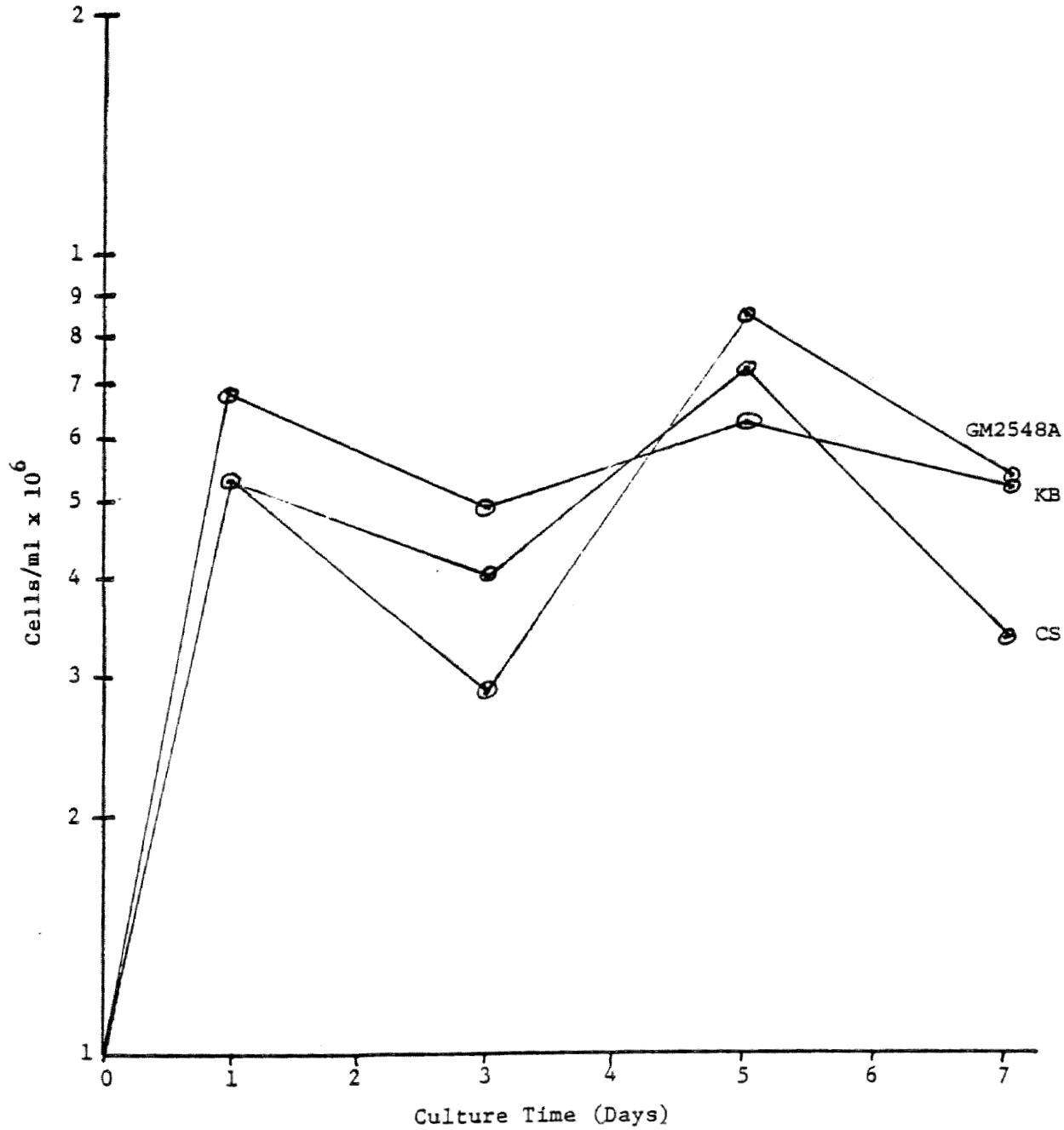

Figure 7. Fibroblast growth curve, Treatment G, Growth supplements: 0.5% fetal bovine serum, 1.0 ng/ml fibroblast growth factor.

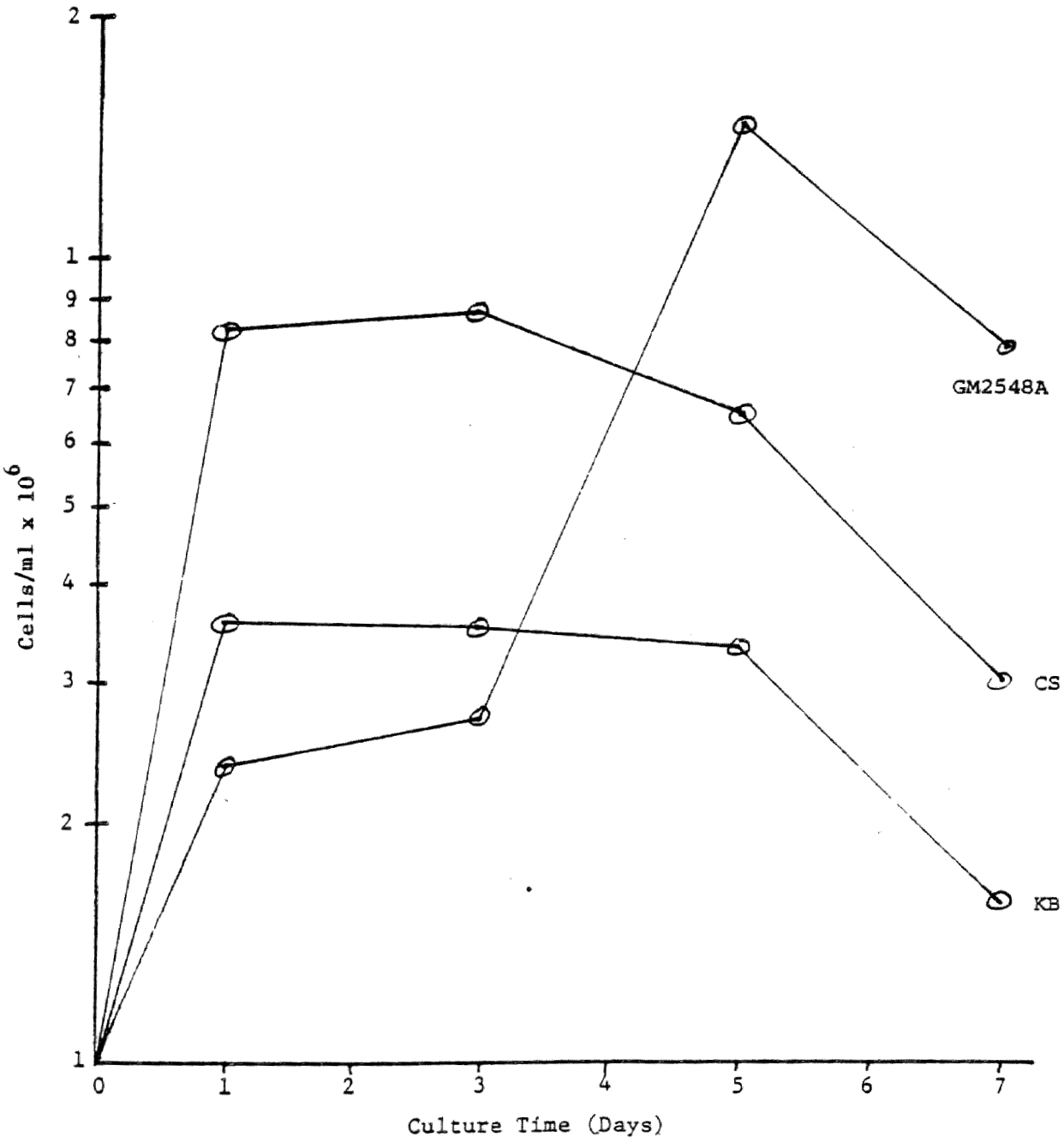

Figure 8. Fibroblast growth curve, Treatment H, Growth supplements: 0.5% fetal bovine serum, 10.0 ng/ml fibroblast growth factor.

flask being counted.

Population doubling time, also called "generation time" was determined for each cell line during the first 48 hours of growth. This time interval was chosen so that comparisons of the cell lines could be made between treatments as well as within a treatment. As a population did not always double during this period of time, a growth rate constant (μ) for each cell line at each treatment level was calculated. This growth rate constant is a proportionality constant that can serve as an index of a culture's rate of growth. It is determined by using the formula,

$$\mu N = \frac{dN}{dt} \quad \text{or} \quad \mu = \frac{d \log_{10} N(2.303)}{dt}$$

where μ = the growth rate constant, N = the number of cells/ml, and t = time. The relationship between μ and the population doubling time (g) for a culture is expressed by the formula,

$$g = \frac{Ln2}{\mu} = \frac{0.693}{\mu} \quad .$$

The values for the proportionality constant ( ) and the population doubling time (g) for each cell line under each of the eight treatments are listed in Table 1.

Table 1. Population Doubling Times and Growth Rate Constants During the First 48 Hours of Culture at Different Growth Supplement Levels

Treatment, Growth Supplement	Cell Line KB μ	KB g	CS μ	CS g	GM2548A μ	GM2548A g
A. Control (No FBS, No FGF)	0.654	1.060	0.615	1.127	0.677	1.024
B. 20% FBS	0.747	0.928	0.824	0.841	0.774	0.895
C. 20% FBS, 0.1 ng/ml FGF	0.532	1.303	0.699	0.991	0.800	0.866
D. 20% FBS, 1.0 ng/ml FGF	0.708	0.980	0.674	1.028	0.427	1.623
E. 20% FBS, 10.0 ng/ml FGF	0.853	0.812	0.741	0.935	0.612	1.132
F. 0.5% FBS, 0.1 ng/ml FGF	0.706	0.982	0.980	0.707	0.634	1.093
G. 0.5% FBS, 1.0 ng/ml FGF	0.879	0.788	0.774	0.895	0.687	1.009
H. 0.5% FBS, 10.0 ng/ml FGF	0.661	1.048	1.085	0.639	0.468	1.481

μ = Growth rate constant
g = Population doubling time (in days)

Maximum cell concentrations reached by each of the cell lines under each of the growth supplement treatments over the seven-day culture period are listed in Table 2. This maximum level of cells/ml cannot be described as a "plateau" level for all cell lines. In some cases, a plateau level was reached and maintained, but in others the culture reached a peak level, then declined. Figure 9 shows plots of the maximum cell concentrations for each cell line in response to tenfold increases in FGF at a high serum level (20% FBS). Figure 10 shows plots of the maximum cell concentrations in response to tenfold increased in FGF at a low serum level (0.5% FBS).

Mitotic index. The mitotic index was determined for each cell line on the basis of number of cells in metaphase division per 1,000 cells counted on the slides prepared for sister chromatid exchange analysis. An average mitotic index was determined from five separate counts. These results are reported in Table 3.

Sister chromatid exchange analysis. Cells from each cell line were cultured for 72 hours in DME medium containing BrdU (30 µg/ml) supplemented with 20% fetal bovine serum and penicillin/streptomycin (100 U/100 µg/ml), then harvested. Slides were prepared and stained for sister chromatid exchange analysis. The counts of these sister chromatid exchanges for each cell line are recorded in

Table 2. Maximum Cell Concentrations at Different Growth Supplement Levels.

Treatment, Growth Supplement	Maximum Cell Concentration (cells/ml x 10^6) KB	CS	GM2548A
A. Control (No FBS, No FGF)	7.50	6.35	4.08
B. 20% FBS	6.65	6.11	5.60
C. 20% FBS, 0.1 ng/ml FGF	4.45	7.98	6.40
D. 20% FBS, 1.0 ng/ml FGF	9.90	10.35	11.35
E. 20% FBS, 10.0 ng/ml FGF	9.25	10.98	12.80
F. 0.5% FBS, 0.1 ng/ml FGF	4.52	8.03	3.95
G. 0.5% FBS, 1.0 ng/ml FGF	6.90	7.62	8.95
H. 0.5% FBS, 10.0 ng/ml FGF	3.92	8.91	15.80

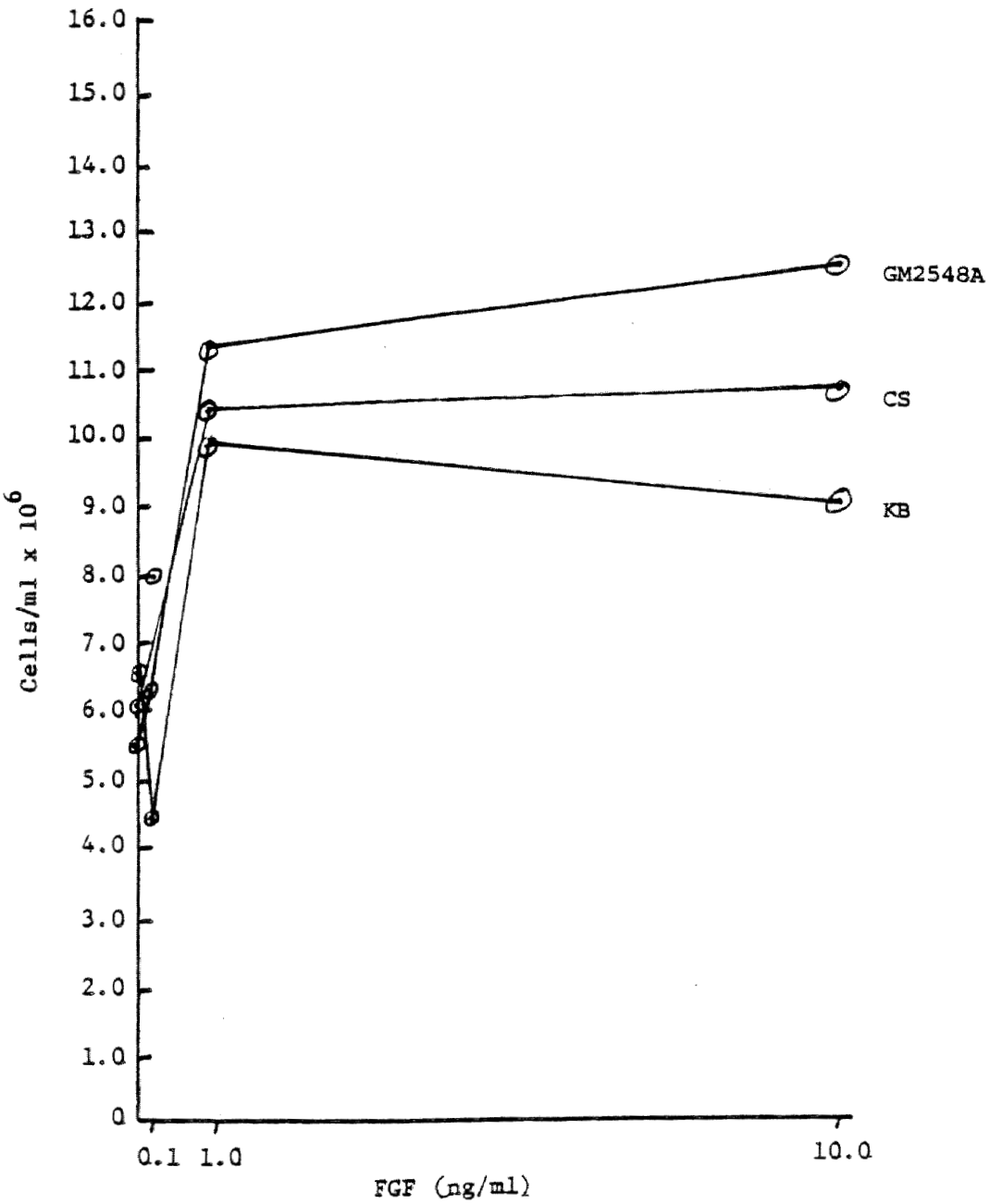

Figure 9. Maximum cell concentrations with tenfold increases of FGF (20% FBS).

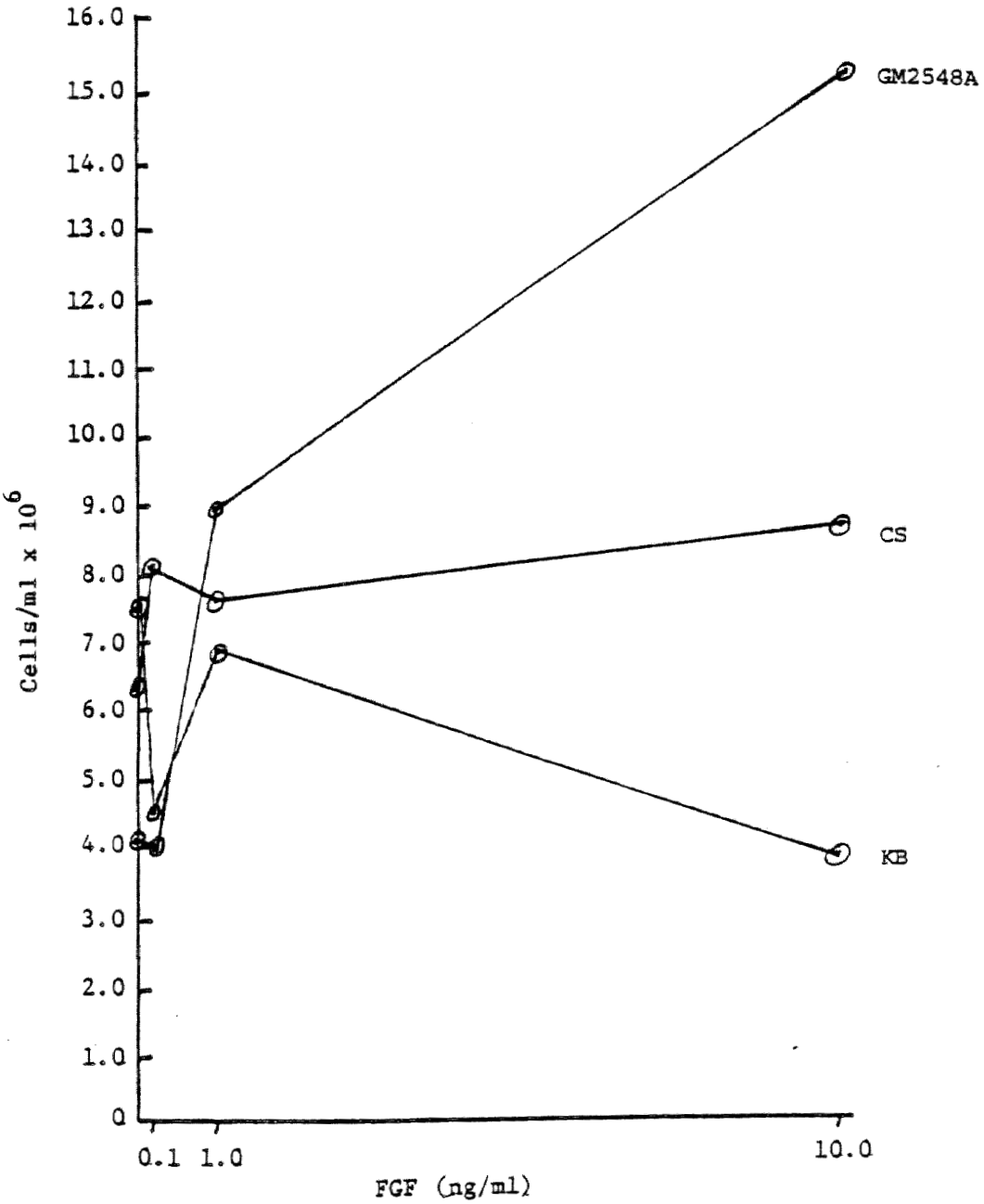

Figure 10. Maximum cell concentrations with tenfold increases of FGF (0.5% FBS).

Table 3. Average Mitotic Index.

Cell Line	Cells in Division/1000 Cells
CS	4.0 ± 0.45
GM2548A	3.0 ± 0.37
KB	0.6 ± 0.18

Table 4. Sister Chromatid Exchange Levels.

Cell Line	Number of Cells	Range of SCE's	Mean SCE	Standard Error
CS	10	4 - 17	8.89	± 0.40
GM2548A	5	41 - 86	60.75	± 3.77
KB	5	3 - 15	8.25	± 1.02

Table 4. The normal cell line, CS, yielded the most metaphases for analysis, with respect to both chromosome spreading and chromatid differentiation. Ten complete cells from this line were chosen for SCE analysis. Neither the Bloom Syndrome cell line, GM2548A, nor the KB cell line yielded as many comparable metaphase spreads. Often the chromosome spreads were incomplete, involved many overlaps, were too contracted, or were first-division metaphases, making them unsuitable for analysis. For these reasons, as well as lower mitotic indices for these lines compared with the CS line, fewer cells in these lines were used for sister chromatid exchange analysis.

DISCUSSION

Growth response to FGF. Population doubling time (Table 1). Measurement of population doubling times during the first 48 hours of culture showed all three cell lines to exhibit similar rates of growth under the control conditions. When cultured in medium without the addition of any growth supplements (Treatment A), all three lines doubled their populations in slightly over one day, the normal cells (CS) responding a little more slowly (1.13 days) than the KB and GM2548A lines. When cultured in medium supplemented with 20% FBS (no FGF, Treatment B), all three lines doubled their populations in just under one

day's time, the normal cells (CS) responding slightly better (0.84 days) to the addition of serum.

When FGF, in tenfold increases, was added to medium with 20% FBS (Treatments C, D, and E), some differences in the growth rates emerged. At the lowest concentration of FGF (0.1 ng/ml, Treatment C), the KB cells were the slowest responders, doubling their population in 1.3 days. The Bloom Syndrome cells (GM2548A) responded best (0.87 days) at this level, although their rate of growth was approximately the same as that seen in Treatment B (no FGF). The normal cell line (CS) doubled its population in just under one day (0.99 days). Thus, this level of FGF cannot be said to enhance the growth of any of the cell lines and it appears to have retarded the growth of the KB line slightly. These results support the findings of Gospodarowicz et al. (1983) in which pituitary FGF in medium supplemented with 20% fetal bovine serum was not active below the level of 0.25 ng/ml. Treatment D (1.0 ng/ml FGF) produced population doubling rates similar to those of the control treatments in both the KB and CS cell lines, but retarded the growth in the GM2548A cell line (1.62 days). In spite of this, GM2548A was shown to have the highest cell concentration of the three lines over the culture period (Figure 4). The highest concentration of FGF at 20% serum (10.0 ng/ml, Treatment E) again showed a population doubling time of just under one

day for the normal cell line. Thus, it appears that the normal fibroblasts were unaffected by the addition of FGF at any of the three concentrations tested in the presence of a high level of serum. Some reduction in population doubling time was expected, as Gospodarowicz and Moran (1975) found that FGF at a concentration of 25 ng/ml in medium with 20% FBS reduced the population doubling time of normal human fibroblasts by half. It is possible that a concentration of 10 ng/ml FGF was too low for this effect to be seen, or that the CS cells used the growth factors in the serum preferentially, not utilizing the purified FGF. The KB cells showed the lowest generation time (0.81 days) under Treatment E, 13% lower than the generation time in the culture with 20% serum alone. Thus, at this treatment level, the KB line demonstrated a positive growth response to FGF, with an additive effect of the serum and FGF suggested. The GM2548A cell line had a population doubling time of 1.13 days with Treatment E, a slight retardation of the growth rate. This line, however, again had the highest cell concentration overall for this treatment (Figure 5). It would appear that the addition of 1.0 ng/ml and 10.0 ng/ml amounts of FGF to medium with 20% FBS had the effect of slightly retarding the initial generation time in this Bloom Syndrome cell line. This lengthening of the lag time could reflect a period of adjustment by these cells in

preparation for their eventual utilization of the FGF.

Mammalian cells in tissue culture have been shown to require serum for growth and when serum is absent or present at low concentrations (< 1%), growth is limited (Gospodarowicz and Moran 1975). The growth-promoting factors in serum remain mostly unidentified, but there has been some evidence that steroids and polypeptides in serum are required components (Gospodarowicz and Moran 1975). The singular effect of the polypeptide FGF can, thus, best be tested by its addition to cultures in the presence of a minimal amount of serum. When FGF was added, in tenfold increases, to medium containing only 0.5% FBS (Treatments F, G, and H), the results in terms of population doubling time were mixed. The data show that all three concentrations of FGF in medium with low serum enabled cultures of the KB and CS lines to maintain or reduce their generation times relative to those seen in cultures with 20% serum (Treatment B). Despite the fact that these lines showed only a small serum response, their generation times at a 20% serum concentration being just slightly under the control (no serum, no FGF) generation times, the data suggest that KB and CS cells were able to utilize FGF effectively for growth. In the normal cell line (CS), FGF was able to reduce the population doubling times at two of the treatment levels (0.1 ng/ml and 10.0 ng/ml) below those seen at the

20% FBS levels, and in the KB line, a reduction was seen at one treatment level (1.0 ng/ml). Even though these generation time reductions were not seen uniformly in each line, they still suggest that FGF, by itself, could enhance the population doubling rates of CS and KB. It is possible that these cells were best able to use this growth supplement in its purified form. The Bloom Syndrome cell line (GM2548A) exhibited retardation of the population doubling time (1.48 days) in response to FGF at 10.0 ng/ml with low serum, and doubling times similar to the control (Treatment A) rate at the other two FGF levels. Taken by itself, this effect would possibly indicate a serum-dependence of the line. The fact that growth retardation was seen at both high and low serum levels, however, and especially at the higher concentrations of FGF, suggests a growth-delaying action of FGF with respect to this cell line. The effect cannot be called inhibitory because each of the growth-retarded cultures ultimately achieved the highest cell concentration for that particular treatment (Table 2). The delayed initial growth could be due to the cells' need to switch to an alternate pathway for the utilization of purified FGF. Also, because GM2548A was a longer-passage cell line compared with the other two cell lines, it is possible that cell selection had occurred so as to create a serum-preference in the line, if not a

serum-dependence.

It should be noted that slight retardations or enhancements of the generation times seen in any of the cell lines at a particular treatment level could be due to chance variations. Such variations could result from counting errors or differences in the initial cell densities of the flasks. Repeated measurements of each cell population using a Coulter counter tend to minimize these potential sources of error.

Growth response to FGF. Maximum cell concentrations. The maximum cell concentrations reached over the seven-day culture period by the three cell lines reveal patterns of growth response to fibroblast growth factor (Table 2). The Bloom Syndrome cell line (GM2548A) exhibited a progressive response to increasing levels of FGF, at both high and low FBS concentrations in the medium. The maximum cell concentration for this line increased 27% over that seen at the control level (Treatment A) upon the addition of 20% FBS (Treatment B). Increasing amounts of FGF in conjunction with this level of serum led to corresponding increases in maximum cell concentrations. At 1.0 ng/ml and 10.0 ng/ml FGF, the Bloom Syndrome line responded with maximum cell levels more than two times that seen with 20% serum alone. A low serum level (0.5% FBS) with a minimal amount of FGF (0.1 ng/ml) produced no growth

in GM2548A above that of the control (Treatment A) level. The two higher concentrations of FGF at low serum, however, produced corresponding changes in maximum cell levels. At 1.0 ng/ml and 0.5% FBS, the culture reached a cell maximum two times greater than that of the control (Treatment A) level, and at 10.0 ng/ml FGF and 0.5% FBS, the level was four times that of the control. These data demonstrate that the Bloom Syndrome cells were sensitive to fetal bovine serum and fibroblast growth factor both singly and in combination with respect to maximum cell concentration, with the greatest effect being seen at the highest level of FGF at both serum concentrations.

The normal cell line (CS) also displayed a growth response to FGF. Control and 20% FBS cultures (Treatments A and B) produced similar maximum cell concentrations, indicating a lack of serum dependency for the line. The addition of FGF to medium with 20% serum resulted in successive increases in the maximum cell concentrations, though the increase between the 1.0 ng/ml and 10.0 ng/ml FGF levels was slight. This suggests that the cell line had reached a plateau level of response to FGF, an idea which is supported by the research of Gospodarowicz et al. (1983). In testing the effect of FGF on human endothelial cells, these researchers found bovine pituitary FGF in medium with 20% serum to be saturating in its effect at 2.5 ng/ml.

Although the data from Treatments A and B imply that CS has a minimal serum response, a slightly additive effect was seen with the addition of FGF to medium with 20% serum. In conjunction with low serum, the additions of all three concentrations of FGF produced maximum cell concentrations in the CS culture equal to or higher than that seen at 0.1 ng/ml and a high serum level, and surpassing that seen with 20% serum alone. These growth responses suggest that the CS line is sensitive to fibroblast growth factor, alone.

The KB cell line responded to FGF plus 20% FBS in a manner similar to that of the normal control line, although the response was uniformly weaker. Like CS, the KB line exhibited similar maximum cell concentrations with both Treatments A and B, the cells responding slightly and inexplicably better to the medium lacking serum. Like CS, this implies a lack of serum dependency. The addition of FGF to medium with 20% serum elicited a growth response in KB at the 1.0 ng/ml and 10.0 ng/ml levels. Again, these maximum cell concentrations were shown to reach a plateau, although at a lower level than that attained by the CS cells. Still, the maximum cell concentration at this plateau level was 1.5 times the maximum concentration achieved by the culture with 20% serum alone, showing that FGF with high serum did have a growth stimulating effect on this line, too. This growth response at the two highest FGF

levels in medium with 20% serum imply a slight additive effect of the growth supplements, the two having to work in combination to effect a growth increase. The maximum cell concentrations seen when FGF was added in conjunction with 0.5% FBS, however, pointed up a difference between this cell line and the others. At 0.1 ng/ml and 10.0 ng/ml FGF, the maximum cell concentrations fell below that of the control level (Treatment A), while at 1.0 ng/ml FGF, the maximum cell concentration was comparable to the control level. These data show that FGF by itself did not stimulate growth of the KB cell line. This could be due to a lack of cell machinery able to effectively utilize FGF by itself, or in its purified form.

In measuring the effects of FGF on early passage human fibroblasts, Gospodarowicz and Moran (1975) found that FGF at 10.0 ng/ml stimulated DNA synthesis as effectively as did an optimal amount of serum. Using this as a point of comparison, the CS cell line was seen to support this finding, utilizing 10.0 ng/ml FGF even a little more effectively for growth than it did the 20% serum. The Bloom Syndrome cell line surpassed this expectation, nearly tripling the maximal growth level of the 20% serum treatment. The KB cells, on the other hand, did not exhibit this response and could even be termed "defective" in their ability to utilize FGF.

Figures 9 and 10 compare the responses of the three cell lines to FGF with high and low serum levels, respectively. Figure 9, the plot of the maximum cell concentrations attained with FGF in medium with 20% FBS, demonstrates that GM2548A was the cell line to respond most positively to FGF, CS gave the second best response, and the response of KB was the weakest. All three cell lines attained a plateau level of growth between the 1.0 ng/ml and 10.0 ng/ml concentrations of FGF, supporting the findings of Gospodarowicz et al. (1983) relative to a saturation effect of FGF. Figure 10, the plot of the maximum cell concentrations attained with FGF in medium with 0.5% FBS, shows the strong growth response of the GM2548A line to FGF alone, the intermediate response of the CS line, and the non-response of the KB line. The CS line again seemed to reach a plateau between 1.0 ng/ml and 10.0 ng/ml FGF concentrations, but the GM2548A line exhibited a steady increase in growth between these two levels. The KB line showed a general decline in growth. It is possible that, because the Bloom Syndrome line was a late-passage cell line, cell selection had taken place to the point where those cells best able to respond to the addition of FGF had been retained, resulting in a more homogeneous and better-responding population. The early-passage lines, CS and KB, most likely represent more heterogeneous cell populations

which might be less able to respond overall.

It should be noted that the measurement of maximum cell concentrations is a measure of a peak level that was often followed by a period of decline in each of the cell lines. Such a period of population decline is due to cell death and can result from an accumulation of acid by-products in the medium or contamination of the culture. Regular changes of the culture medium should reduce the accumulation of toxic cell products. Visual indications of contamination were not present; however, hard-to-detect viral or mycoplasmal contamination could have been present.

Mitotic index. Table 3 shows the normal cell line, CS, to have the highest average mitotic index of the three cell lines (4±2.24). The Bloom Syndrome cell line, GM2548A, had the second highest mitotic index (3±1.87). The KB line exhibited the poorest growth in terms of this measurement, with a mitotic index of 0.6±0.89. These data point up the much lower growth rate of the KB cell line compared with that seen in both normal and Bloom Syndrome cells and support the findings of Brat (1979).

Sister chromatid exchange analysis. The average numbers of sister chromatid exchanges per metaphase for each cell line are listed in Table 4. These averages are based on sister chromatid exchanges counted in five cells for the GM2548A and KB cell lines, and ten cells for the CS cell

line. Because of incomplete metaphases, chromosome crowding, and overlaps, a smaller number of suitable metaphases was available for analysis in the Bloom Syndrome and KB lines. Nevertheless, the data clearly indicate that the average number of sister chromatid exchanges in the KB cells (8.25±1.02) corresponds to the average number seen in the normal cell line (8.89±0.40) and falls within the expected range of average values for normal cells (6.9 - 9.3) (Chaganti et al. 1974; German et al. 1977a). The Bloom Syndrome cell line exhibited an average sister chromatid exchange level that was predictably high (60.75± 3.77) and characteristic for Bloom Syndrome (Chaganti et al. 1974).

These findings indicate that the KB fibroblastic cell line does not display the characteristic cytogenetic features associated with Bloom Syndrome with respect to sister chromatid exchange. The average number of sister chromatid exchanges in the KB fibroblasts and their range (3-15) correspond to similar values seen previously in the KB lymphocytes (mean, 14; range, 4-21) (James 1984, unpublished data). The differences in the means is possibly due to the small sample size of the fibroblastic cells. Thus, the sister chromatid exchange level was shown to be in the normal range of KB fibroblastic cells as well as for lymphocytes, which supports previous findings of

consistencies in the sister chromatid exchange levels between lymphocytes and fibroblasts in both normal (Zakharov 1982) and Bloom Syndrome cells (Brat 1979). The fact that the finding of two populations of lymphocytes with respect to sister chromatid exchange levels, reported in some Bloom Syndrome patients, has never been seen in fibroblasts (Shiraishi et al. 1983b) suggests that the sister chromatid exchange level determined for the KB fibroblasts is an accurate representation of the patient's sister chromatid exchange rate.

CONCLUSIONS

The diagnosis of Bloom Syndrome in the KB patient is not supported on the basis of the growth responses and sister chromatid exchange rate determined for the fibroblastic cells. It is possible that the patient has a variation of Bloom Syndrome not previously reported in which the clinical features of the syndrome are present, but the cellular responses are atypical when compared to classical Bloom Syndrome. Variations of this sort are known for another chromosome fragility disorder, xeroderma pigmentosum, for which seven complementation groups have been reported (Witkop 1983). It is also possible that the case represents a disorder other than Bloom Syndrome, in which case different criteria would have to be used to establish a prognosis for this patient. In particular, the

expectation for cancer predisposition may be reduced because of the normal sister chromatid exchange rate observed in both lymphocytes and fibroblasts.

SUMMARY

This research compared features of the KB cell line of a patient who had been diagnosed with Bloom Syndrome with a known Bloom Syndrome cell line, GM2548A, and a normal control cell line, CS, with respect to growth response to fibroblast growth factor, mitotic index, and sister chromatid exchange rate.

Measurements were made of population doubling time and maximum cell concentration produced in response to three levels of fibroblast growth factor in combination with high and low levels of fetal bovine serum. The KB cell line demonstrated that, like the normal cell line, it could use FGF for growth as measured in terms of population doubling time. In terms of maximum cell concentration achieved over the culture period, however, KB showed the weakest response to FGF in medium with high FBS, and no response to FGF in medium with low FBS. The KB cell line also showed a minimal response to serum in medium. With both measurements, the responses of the KB cells were dissimilar to those of the known Bloom Syndrome cells. The mitotic index for each cell line demonstrated that the KB cell line grew much more slowly than the other two lines, as the KB mitotic index was

considerably lower. Analysis of sister chromatid exchange rates showed that the average number per metaphase for the KB fibroblasts was within normal values, and unlike the elevated rate seen in the Bloom Syndrome cells. These combined observations suggested that the KB fibroblastic cells are impaired, differing from both the normal and Bloom Syndrome cells with respect to growth response to fibroblast growth factor and mitotic index, but resembling normal cells with respect to sister chromatid exchange rate.

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